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REGULATION OF PHOSPHOLIPASE ACTIVITY IN POTATO LEAVES BY CALMODULIN AND PROTEIN PHOSPHORYLATION-DEPHOSPHORYLATION

High levels of phospholipase activity were measured in potato (*Solanum tuberosum* L. cv. Kennebec or Russett Burbank) leaf extracts using a new fluorometric phospholipase assay based on 1-acyl-2-[6-[(7-nitro-2,1,3 benzoxadiazol-4-yl)amino]-caproyl] phosphatidylcholine (C_6-NBD-PC). Time-course studies revealed that phospholipase activity could be stimulated for a brief time by the addition of calmodulin or the catalytic subunit of cyclic AMP-dependent protein kinase. The short-lived calmodulin stimulation or protein kinase stimulation of phospholipase activity could be prolonged by either conducting the time-course reactions in the cold (5°C) or adding sodium fluoride (a phosphatase inhibitor) to the reaction mixtures. Centrifugation studies revealed that calmodulin-stimulated or protein kinase-stimulated phospholipase activities were soluble and not associated with membranes. When potato leaves were homogenized in the presence of either of two phosphatase inhibitors, the levels of phospholipase activity in the corresponding high-speed supernatant fractions were 36—47% higher than in controls. These experiments suggest a possible protein phosphorylation-dephosphorylation mechanism for the regulation of phospholipase activity in potato leaves.

Introduction

Potato leaves and tubers contain very high levels of lipolytic acyl hydrolases [1,2]. These enzymes cause the rapid hydrolysis of endogenous phospholipids during homogenization and organelle isolation. We recently reported that calcium and calmodulin stimulate this autolytic process in leaf homogenates [3]. The present study was undertaken in order to further characterize this newly reported calmodulin effect. Because calmodulin has been reported to stimulate many enzymes indirectly, via calmodulin-dependent protein kinases [4], part of this study was designed to investigage whether calmodulin may regulate the lipolytic enzymes in potato leaves by this type of protein phosphorylation-dephosphorylation mechanism.

Abbreviations: BSA, bovine serum albumin; C_6-NBD-PC, 1-acyl-2-[6-[(7-nitro-2,1,3 benzoxadiazol-4-yl)amino]-caproyl]phosphatidylcholine; HEPES, *N'*-2-hydroxy-ethylpiperazine-*N*-2-ethanesulfonic acid; PC, phosphatidylcholine.

Materials and methods

Materials

Seed potato tubers (*Solanum tuberosum* L. cv. Kennebec or Russett Burbank) were planted in 6-inch clay pots in commercial potting soil. Plants were grown in a greenhouse with continuous supplemental light from four 34-W cool white fluorescent lamps. Calmodulin (bovine brain), the catalytic subunit of cyclic AMP-dependent protein kinase, and ATP were obtained from Sigma. C_6-NBD-PC (see below) was obtained from Avanti Polar Lipids, Birmingham, AL, U.S.A. All other reagents were the best grades commercially available.

Preparation of subcellular fractions

Potato leaves (5 g of leaves 1—2 cm in length) were rinsed with distilled water and homogenized in a chilled mortar and pestle with 20 ml of a solution containing 0.3 M sucrose, 0.1 M *N*-2-hydroxy-ethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (pH 7.5), 5 mM dithiothreitol, and 5 mM β-mercaptoethanol. When noted, various potential inhibitors were dissolved in the grinding media before homogenization. The homogenate was filtered through two layers of cheesecloth and centrifuged at 13 000 × *g* for 20 min. The 13 000 × *g* supernatant fraction was removed and part of it (10 ml) was subjected to centrifugation at 100 000 × *g* for 50 min. The 100 000 × *g* supernatant fraction was removed and the 100 000 × *g* pellet was resuspended in 10 ml of the above grinding medium. All procedures were conducted at 0—4°C.

Time-course studies

One milliliter of the 13 000 × *g* or 100 000 × *g* supernatant fraction, or 100 000 × *g* pellet (resuspended as described above), was added to a small test tube and 0.1 ml of a solution containing the various test compounds (Mg^{2+}, ATP, calmodulin, protein kinase, etc.) was added. The mixture was shaken at 120 rev./min in a water bath at either 5°C or 25°C. At various times small samples (10—50 μl) were removed and immediately assayed for phospholipase activity.

Phospholipase assay with C_6 NBD PC

Phospholipase activity was assayed by a fluorometric technique which was recently reported [6]. This assay employs the fluorescent phospholipid analogue, C_6-NBD-PC as a substrate. When the ester bond in the 2 position is cleaved, the relative fluorescence increases 50-fold. To a fluorometer cuvette was added a reaction mixture (2 ml) containing 5 μM C_6-NBD-PC, 50 mM HEPES buffer (pH 7.1), and 10—50 μl of enzyme. Relative fluorescence was measured continuously (5—10 min/sample) with a recorder attached to a Sequoia-Turner Model 450 Fluorometer equipped with an excitation filter (460 ± 5 nm) and an emission filter ($\geqslant$535 nm). A calibration curve of fluorescent product was constructed using chemically hydrolyzed C_6-NBD-PC (hydrolyzed with 1.5 N methanolic KOH heated to 70°C for 30 min, and then neutralized to pH 7.1). Protein was assayed as previously described [5].

Results

In our previous report [3], we demonstrated that the rate of autolysis of endogenous phospholipids in potato leaf homogenates was stimulated by calmodulin. For the present study a new fluorometric phospholipase assay [6] was investigated because it offered the advantage of being able to detect rapid changes in phospholipase activity. Using this assay, C_6-NBD-PC was hydrolyzed at a rate of about 2 nmol min^{-1} ml^{-1} of 13 000 × *g* supernatant fraction at 25°C and pH 7.1. In the previous study [3], we reported that 14.3% of the endogenous phosphatidylcholine (PC) in potato leaf homogenates was hydrolyzed in 30 min at 25°C (the final pH of the homogenates was also about 7.1). The concentration of PC in the crude homogenates was about 200 nmol/ml. The rate of hydrolysis of endogenous PC can then be calculated to be 0.95 nmol min^{-1} ml^{-1} homogenate. Since PC comprises about 50% of the phospholipids in potato leaf homogenates [7] the total rate of autolysis of phospholipids would be close to 2 nmol min^{-1} ml^{-1} homogenate. Based on these very similar rates of hydrolysis of endogenous PC and synthetic PC analogue (C_6-NBD-PC) it seemed reasonable to employ this new fluorometric assay [6] in the present study.

In the first time-course experiment Fig. 1A) the phospholipase activity in the 13 000 × *g* supernatant fraction remained nearly constant for 60 min at 25°C. The addition of 3 mM Ca^{2+} caused a slight stimu-

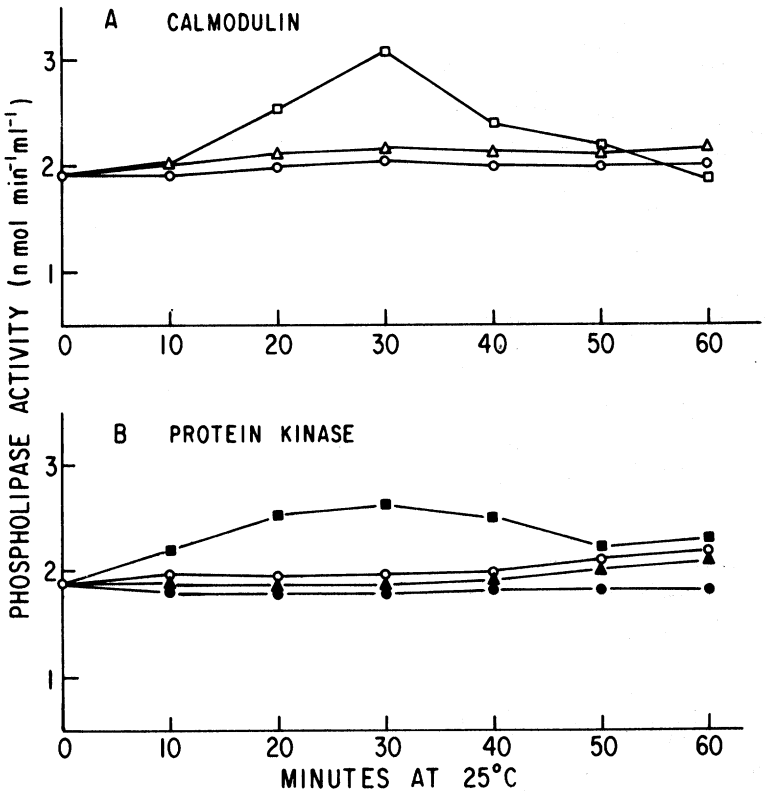

Fig. 1. Time-course study of the stimulation of phospholipase activity in the 13 000 × *g* potato leaf supernatant fraction at 25°C by (A) calcium and calmodulin and (B) the catalytic subunit of cyclic-AMP dependent protein kinase. Control (0) = 13 000 × *g* supernatant; same plus 3 mM Ca^{2+} (△); same plus 3 mM Ca^{2+} and calmodulin (10 000 units/1.1 ml) (□); same plus 3 mM Mg^{2+} (●); same plus 3 mM Mg^{2+} and 0.3 mM ATP (▲); same plus 3 mM Mg^{2+}, 0.3 mM ATP and protein kinase (500 units/1.1 ml) (■) (all final concentrations).

lation of activity. The subsequent addition of 1 μM calmodulin and 3 mM Ca^{2+} caused a 50% stimulation in phospholipase activity at 30 min but the activity returned to the original level by 50 min. In a similar experiment (Fig. 1B) the addition of magnesium and ATP had no effect on phospholipase activity, but the subsequent addition of the catalytic subunit of c-AMP-dependent protein kinase caused a 30—40% stimulation of phospholipase activity for a short period (20—40 min). Control experiments showed that neither of the commercially prepared proteins (calmodulin or c-AMP-dependent protein kinase) hydrolyzed the fluorescent substrate. The similarities in degree and shape of the calmodulin-dependent stimulation (Fig. 1A) and protein kinase-dependent stimulation (Fig. 1B) of phospholipase activity prompted further investigation.

Two techniques were used to try to stabilize the stimulated form of the phospholipase activity. In the first (Figs. 2A & B), time-courses were conducted at 5°C instead of 25°C. As anticipated, the calmodulin stimulation (about 20—30%) and the protein kinase stimulation (about 30—40%) were maintained at 5°C for up to 4 h. The levels of stimulation which are illustrated in Fig. 2 were maintained for up to 12 h (data not shown) at 5°C, and then samples began to turn brown and all enzyme activities declined.

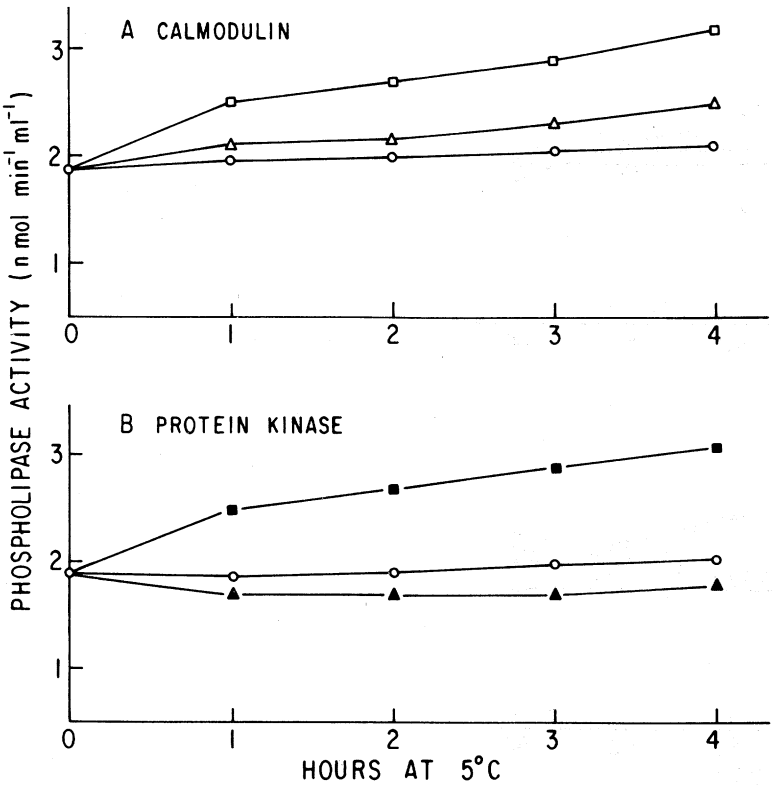

Fig. 2. Time-course study of stimulation of phospholipase activity in the 13 000 × *g* potato leaf supernatant fraction at 5°C by (A) calcium and calmodulin and (B) the catalytic subunit of cyclic-AMP dependent protein kinase. For key to symbols see Fig. 1.

The cold temperature thus appeared to inhibit the enzymes (perhaps phosphatases) that may have caused the rapid breakdown of the stimulated form of the enzyme shown in Fig. 1A and B. In our previous report [3], the degree of calmodulin stimulation of the autolytic hydrolysis of PC was also comparable at 4°C and 25°C. The second technique (Fig. 3A & B), was to try to stimulate the phospholipase in the presence of a phosphatase inhibitor (50 mM sodium fluoride) at 25°C. As in Fig. 1B, phospholipase activity showed a slight increase in the presence of Mg^{2+} plus ATP alone (NaF alone also gave a slight increase), but surprisingly when 50 mM NaF was combined with Mg^{2+} and ATP the activity increased at a nearly constant rate during the first 50 min. The further addition of either protein kinase (Fig. 3A) or calmodulin (Fig. 3B) stimulated phospholipase activity to its maximal level at 20 min and 30 min, respectively, and then the activities declined slowly for the remainder of the experiment. The fluoride treatment was therefore successful in preventing the sharp decline in phospholipase activity which occurred in Fig. 1. Although protein kinase and calmodulin caused phospholipase activity to reach its maximal value at earlier times, the actual maximum value was nearly the same with (a) NaF, Mg^{2+}, and ATP, (b) NaF, Mg^{2+}, ATP, and protein kinase, (c) NaF, Mg^{2+}, ATP, and Ca^{2+}, and (d) NaF, Mg^{2+}, ATP, Ca^{2+}, and calmodulin. Thus, it appears that the 13 000 × *g* supernatant fraction contains the endogenous biochemi-

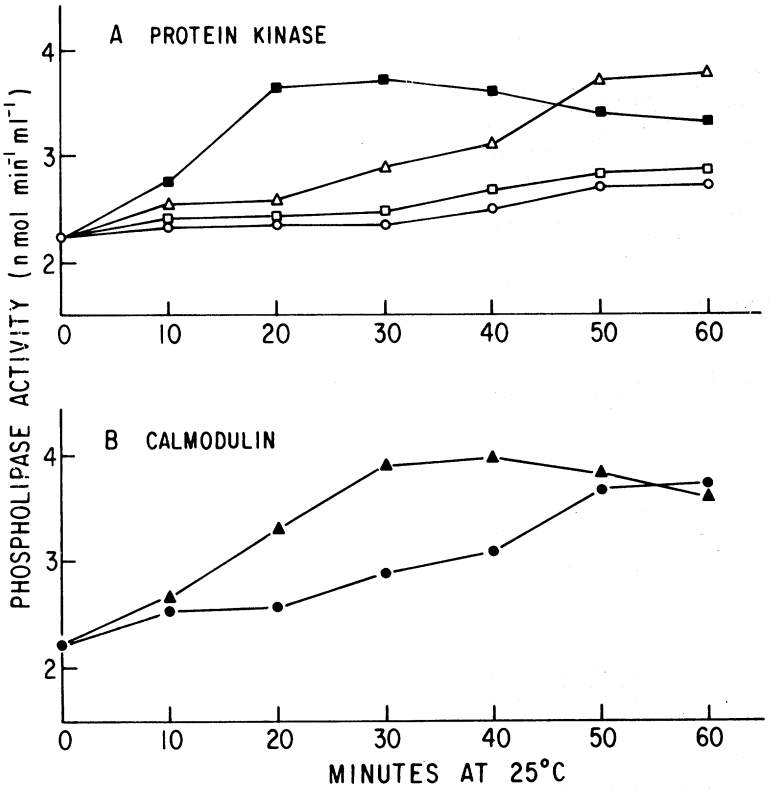

Fig. 3. Time-course study of stimulation of phospholipase activity in the potato leaf 13 000 × *g* supernatant fraction by (A) the catalytic subunit of cyclic AMP-dependent protein kinase and (B) calmodulin at 25°C in the presence of a phosphatase inhibitor (50 mM NaF). Control (○) = 13 000 × *g* supernatant fraction containing 3 mM Mg^{2+} and 0.3 mM ATP; same plus 50 mM NaF (□); same plus 3 mM Mg^{2+}, 0.3 mM ATP and 50 mM NaF (△); same plus 3 mM Mg^{2+}, 0.3 mM ATP, 50 mM NaF and protein kinase (500 units/1.1 ml) (■); same plus 3 mM Mg^{2+}, 0.3 mM ATP, 50 mM NaF and 3 mM Ca^{2+} (●); same plus 3 mM Mg^{2+}, 0.3 mM ATP, 50 mM NaF, 3 mM Ca^{2+} and calmodulin (10 000 units/1.1 ml) (▲) (all final concentrations).

cal machinery (perhaps protein kinases) to cause a 30—40% stimulation of phospholipase activity in 50 min at 25°C in the presence of NaF.

The next experiment (Fig. 4) was designed to determine whether the calmodulin-stimulated or protein kinase-stimulated phospholipase activities in the 13 000 × *g* supernatant fraction are catalyzed by soluble enzymes or are catalyzed by enzymes associated with membranes (microsomes). When the 13 000 × *g* supernatant fraction was further centrifuged at 100 000 × *g* for 50 min, about 75% of the phospholipase activity was associated with soluble enzymes (100 000 × *g* supernatant fraction) and about 25% of the phospholipase activity was associated with membranes (100 00 × *g* pellet). However, when these two fractions were tested for potential stimulation by calmodulin and protein kinase, only the 100 000 × *g* supernatant fraction (Fig. 4A) responded. It thus appears that calmodulin and protein kinase cause the stimulation of a soluble phospholipase activity.

The biochemical properties of the soluble (100 000 × *g* supernatant fraction) and membrane-associated (100 000 × *g* pellet) phospholipase activities were compared (Table I). Although the two enzymes had

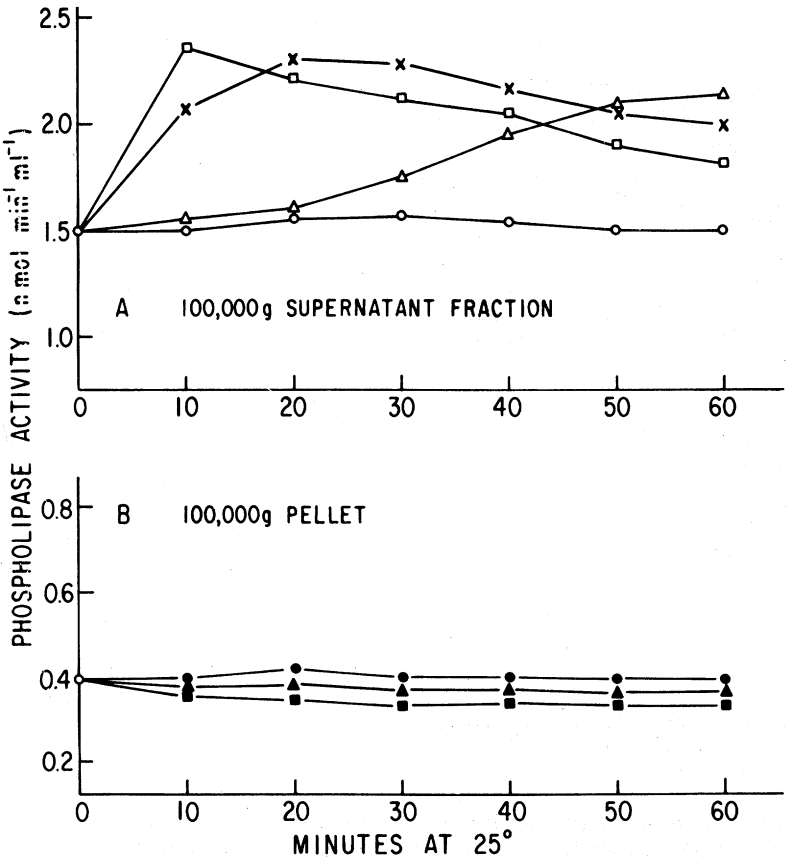

Fig. 4. Time-course study of stimulation of phospholipase activity in (A) 100 000 × *g* supernatant fraction and (B) 100 000 × *g* pellet from potato leaf. Control (○) = 100 000 × *g* supernatant fraction containing 3 mM Mg^{2+} and 0.3 mM ATP; same plus 3 mM Mg^{2+}, 0.3 mM ATP, and 50 mM NaF (△); same plus 3 mM Mg^{2+}, 0.3 mM ATP, 50 mM NaF, and the catalytic subunit of cyclic AMP-dependent protein kinase (500 units/1.1 ml) (□); same plus 3 mM Mg^{2+}, 0.3 mM ATP, 50 mM NaF, and calmodulin (10 000 units/1.1 ml) (X); Control (●) = 100 000 × *g* pellet, resuspended, plus 3 mM Mg^{2+}, 0.3 mM ATP, and 50 mM NaF; same plus 3 mM Mg^{2+}, 0.3 mM ATP, 50 mM NaF, and the catalytic subunit of c-AMP-dependent protein kinase (500 units/1.1 ml) (▲), same plus 3 mM Mg^{2+}, 0.3 mM ATP, 50 mM NAF, and calmodulin (10 000 units/1.1 ml) (■).

similar (but not identical) pH optima and K_m-values for the fluorometric substrate they responded very differently to the various chemical treatments. Even though 6.3 was the optimum pH of the soluble enzyme, the calmodulin and protein kinase stimulated activities were higher when the enzyme was assayed at pH 7.1. The membrane-associated enzyme activity was slightly stimulated by EDTA, completely inhibited by Triton X-100, inhibited (93%) by dibucaine, and showed no response to $CaCl_2$, deoxycholate, or bovine serum albumin (BSA). In contrast, the soluble enzyme activity was stimulated by $CaCl_2$, Triton X-100, and deoxycholate and was inhibited by EDTA, BSA, and dibucaine.

In Fig. 4A the addition of Mg^{2+}, ATP and NaF to the 100 000 × *g* supernatant fraction caused a gradual but constant increase in phospholipase activity during the course of the experiment (increasing to almost 45% by 60 min), similar to that which was described

Table I. A comparative study of the properties of the phospholipase activities in the 100 000 × *g* pellet and 100 000 × *g* supernatant fraction from potato. Results are the mean of two experiments with triplicate samples for each treatment. The various test solutions were added to the cuvette just prior to the addition of enzyme sample and the stated concentrations are those in the final reaction mixture in the cuvette buffered with 50 mM HEPES (pH 7.1) (except for the pH optimum studies which were buffered with 50 mM citrate, MES or tricine, each at several pH values).

Property	100 000 × *g* pellet	100 000 × *g* supernatant fraction
pH optimum	6.0	6.3
K_m for C_6-NBD-PC	1.5 μM	2.2 μM
Effect of 5 mM $CaCl_2$	None	39% stimulation
Effect of 5 mM EDTA	14% stimulation	13% inhibition
Effect of 0.01% Triton X-100[a]	100% inhibition	643% stimulation
Effect of 1 mM sodium deoxycholate	None	55% stimulation
Effect of 25 μg/ml bovine serum albumin[a]	None	61% inhibition
Effect of 0.5 mM dibucaine	93% inhibition	70% inhibition

[a]Higher concentrations interfered with the assay by causing unacceptably high background fluorescence.

with the 13 000 × *g* supernatant fraction in Fig. 3A and B. Thus, the activity in the 100 000 × *g* supernatant fraction also appears to be able to undergo a self-stimulation of phospholipase activity under these conditions. The final experiment (Table II) was designed to investigate whether the addition of phosphatase inhibitors (sodium fluoride and potassium phosphate) to the grinding media would affect the levels of phospholipase activity in the corresponding 100 000 × *g* supernatant fractions. The levels of phospholipase activity in the final supernatant fractions were 37% and 46% higher when phosphate and fluoride, respectively, were present. A sodium chloride treatment was also included as a control and caused only a slight (5%) increase in activity. Since magnesium often stimulates phosphatases [8] a magnesium chloride treatment was also tested and found to have no effect on phospholipase activity. This experiment demonstrates that the composition of the grinding buffer (or even the choice of common phosphate buffer as opposed to organic buffer) can significantly influence the final level of soluble phospholipase activity in the 100 000 × *g* supernatant fractions. The results of this experiment are consistent with a phosphorylation-dephosphorylation mechanism for the regulation of phospholipase activity in potato leaves. Since exogenous ATP was not added in this experiment it appears that the endogenous levels of ATP were adequate. Although it is also possible that sodium fluoride and potassium phosphate merely solubilized enzyme

Table II. Effect of homogenizing potato leaves with various phosphatase inhibitors added to the grinding media. Phospholipase activity was measured in the corresponding 100 000 × *g* supernatant fractions. Number in parenthesis is relative specific activity (no additions = 100%).

Additions to grinding medium (final conc.)	Phospholipase activity	
	Tot. act. nmol min^{-1} ml^{-1}	Spec. act. nmol min^{-1} (mg protein)$^{-1}$
None	0.885	0.079 (100)
100 mM potassium phosphate (pH 7.1)	1.180	0.116 (146)
50 mM sodium fluoride	1.164	0.109 (137)
50 mM sodium chloride	0.913	0.084 (105)
10 mM magnesium chloride	0.953	0.080 (101)

from the membranes or cell walls, the sodium chloride control provides evidence that this is not the case.

Discussion

Prior to this report, there were only four examples of plant enzymes which are modulated by protein phosphorylation-dephosphorylation. They include quinate-NAD oxidoreductase [8], pyruvate dehydrogenase [9], pyruvate P_i dikinase from C_4 leaves [10], and a microsomal H^+-ATPase [11]. This paper presents evidence for the first lipolytic plant enzyme that appears to be regulated by phosphorylation-dephosphorylation.

The 100 000 × *g* supernatant fraction from potato leaves appeared to contain the endogenous components to carry out reversible phosphorylation-dephosphorylation (Fig. 4A). The evidence for phosphorylation was the gradual increase in phospholipase activity in the presence of Mg^{2+}, ATP and NaF. The evidence for dephosphorylation included the gradual decrease of phospholipase activity in the calmodulin and protein kinase treatments and the apparent stimulation of activity when phosphatase inhibitors were added. Several types of protein kinases have been reported in plant materials [4]. They differ in whether or not they are associated with membranes or their degree of stimulation by Ca^{2+}, calmodulin, and phospholipids [12—15]. To date, only one phosphoprotein phosphatase has been reported in plants [16] but it is likely that many of the other plant phosphatases are capable of dephosphorylating phosphoproteins. Although the fluoride effect which was observed in this study is consistent with its role as a phosphatase inhibitor, fluoride is also known to inhibit many other types of enzymes which could either directly or indirectly influence phospholipase activity.

This study provides several pieces of evidence which indicate that the calmodulin stimulation of phospholipase activity is due to the presence of an endogenous calmodulin-dependent protein kinase. The degree of stimulation (20—40%) is comparable after the addition of either calmodulin or protein kinase. The kinetics of stimulation in the various time-course studies are also comparable after the addition of either calmodulin or protein kinase. Two of the other plant enzymes (acutally crude enzyme systems) which have been reported to be regulated by phosphorylation-dephosphorylation also exhibit comparable patterns of stimulation after the addition of calmodulin [11,17]. In order to prove this hypothesis it would be necessary to demonstrate the presence of a soluble calmodulin-dependent protein kinase in potato leaves. Such an enzyme was recently reported in two other plant tissues [14,18]. Although the present evidence is suggestive of an indirect mechanism of calmodulin stimulation via phosphorylation, further work is required in order to discount the possibility of direct binding of calmodulin to a soluble phospholipase(s). Further work is also required in order to evaluate how the regulation of this plant phospholipase affects its activity with endogenous substrates (natural phospholipids and galactolipids).

After this paper was initially submitted for publication Hope et al. [19] presented evidence that several commercial preparations of calmodulin were contaminated by phospholipase A_2. Although they reported a low level of phospholipase activity (0.011 nmol $hour^{-1}$ (mg protein)$^{-1}$) in the same commercial preparation of calmodulin which was used in this study (bovine brain calmodulin prepared by Sigma) phospholipase activity was not detected in it using the fluorometric assay employed in this study.

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